Media Supplements for Improving Ethanol Tolerance and Fermentation Performance of *Saccharomyces cerevisiae*, L1400 (Exp. #3)

Project Title: Amoco CRADA Corn Fiber

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Purpose

To investigate the ability of nutritional additives to enhance the ethanol tolerance **and** fermentation capability of the yeast L1400.

Experimental Design

The original design of this experiment was based on investigating means of increasing the ethanol tolerance of the organism **by** nutrient supplementation and temperature. The literature in this area of research has investigated many additives which reportedly increased the ethanol tolerance of yeast and bacteria. Three additives chosen were phosphatidylcholine/albumin complex, soy flour, and YEP media at twice the standard concentration. Two additional experiments addressing temperature **during** fermentation rather than nutrient supplementation **were** included with the low limit at 30° C and the high limit at 34° C.

Background

There are many factors influencing the final ethanol concentration attained in an ethanol fermentation. Many of the operating conditions have been established for the SSFs in this laboratory and this narrows the options of operating conditions for enhancing ethanol production or tolerance. One approach that has demonstrated measured success is nutrient supplementation. The additives considered in this study was phosphatidylcholine/albumin complex, soy flour, and yeast extract peptone media at twice the recommended concentration.

Phosphatidylcholine/albumin complex as well as soy flour have been shown to increase the ethanol tolerance of yeasts[1-3]. One reason why albumin may be important for increasing ethanol tolerance is that albumin contains the three amino acids phenylalanine, methionine, and tyrosine in large quantities. These amino acids have been shown to be partly responsible for enhanced ethanol productivities **due** to an improvement in the alcohol tolerance of cells[4]. Another factor that may **be** of importance with these additive nutrients is that they supply unsaturated fatty acids **(UFS).** It has been suggested that due to the inability of yeast to produce their own **UFS** under anaerobic conditions this would necessitate the addition of **UFS** in the media'.

The need to have high substrate and ethanol concentrations in an **SSF** presents problems with maintaining healthy yeast populations. This media composition created high osmotic pressure and low water availability that have been shown to inhibit yeast growth and fermentation performance [6,7]. One method for combating these negative effects suggested **doubling** the YEP media concentration. This media alteration reportedly increased ethanol production with an ale brewing strain from 8.5% (w/v) to almost 14% (w/v) ethanol [8].

Materials and Methods

Yeast strain

The organism used in these studies **was** *Saccharomyces cerevisiae* Labatt 1400 strain and **is** a spheroplast fusion product of the polyploid brewing strain *Succharomyces uvarum*, strain **21** and a genetically constructed diploid *Saccharomyces diastaticus*, **strain** 1384[9]. The seed vials were prepared by growing in **YEPD** media for 14 hours then diluting **1/2** with a **40%** (w/v) glycerol solution and **quick** freezing. The vials contained **7** x 10^7 cells/ml. The organism was supplied by Amoco Corporation.

Inoculum Preparation

A one ml frozen vial stored at -70° C was thawed at room temperature and inoculated into 200 ml YEP with **2%** (w/w) glucose media. The inoculum was incubated at 30° ***C*** in a rotary shaker (150 r.p.m.) for **24** hours.

Growth ***Media***

The corn fiber media **was** prepared by adding **2% (w/w)** corn steep liquor (CPN) to pretreated corn fiber. The pretreated corn fiber was prepared by Amoco Corporation. The media was then adjusted to **a** pH of 5.08 that required 25 grams of calcium hydroxide (reagent grade, GFS Chemicals) for 2396 grams of corn fiber.

This standard media was then separated, additional nutrients were added, and the pH adjusted as follows:

Controls

Six flasks were used as controls and loaded with 77.4 **g/l** of this standard media, three **fox** a control at 30° C and three for a control at 34° C. These *six* flasks did not contain any additional media additives and was the same media as the standard media mentioned above.

Yeast Extract and Peptone Media (2X)

The yeast extract/peptone media was prepared at twice the standard concentration. Yeast extract (Difco) was at 2%(w/w) and the peptone (Difco) was at **4%** (w/w). This media required 4.81 ml of sulfuric acid *(5%)* to adjust 300 grams of media to a pH of 5.03. Three flasks were loaded with 77.4 grams of **this** media.

Protective Agent Media

The protective agent was composed of a 2/1 ratio (5/2.5 grams) of albumin/ phosphatidylcholine. The albumin was from Sigma **(A-5253)** and processed from chicken egg whites. The phosphatidylcholine was from **Sigma** (P-9671) and was processed from fresh frozen egg yolks. These ingredients were weighed out and mixed together with a mortar and pedestal. Then 295.5 grams of the corn fiber media was **mixed** with 4.5 grams of the protective agent **(PA)** to give a 1.5%(w/w) concentration. The resultant media pH was then adjusted to a pH of 5.00 that required **1.39** ml sulfuric acid ***(5%)*** to adjust 300 grams media. Three flasks were loaded with **77.4** grams of the final media.

*Soy Flour **Media***

The soy flour media consisted of 6% (w/w) soy flour (Sigma, **S-9633)** and the standard corn fiber media mentioned above. **282** grams corn fiber was mixed with **18** grams **soy flour** and the pH adjusted to 5.01 with **2.65** ml sulfuric acid ***(5%).*** Three flasks were loaded with 77.4 grams of the final media.

Sterilization and Preinoculation Preparation

The fifteen flasks were autoclaved at 121° C for 15 minutes. After cooling, **0.82** ml (500 units or 200 units/g oligomeric **sugar)** amyloglucosidase **(Sigma)** and 0.75 ml (62 IFPU or 20 IFPU/g cellulose) cellulase enzyme (Iogen) was added to each flask, mixed well, **and** incubated for **24** hours. The final weight of the media was 100 grams in each flask after the inoculation step (see below).

Inoculation

Prior to inoculation, 10.1 ml of 200 proof ethanol and **2.5 ml** deionized water was added to each flask. After **mixing** thoroughly, 10 ml inoculum was added to each flask, mixed, and sampled. The final ethanol concentration for all flasks was **8%** (w/w).

Growth Conditions

The SSFs were carried out at 30° C, excluding the three control flasks at 34° C, in 250 ml baffled shake flasks capped with anaerobic gas locks. Triplicates for each media type were fermented in a rotary shaker set at 150 r.p.m.

Analytical techniques

Glucose and ethanol concentrations were determined using a Hewlett Packard 1090 HPLC equipped with a 1047 IR detector and a HPX-87H column. Column temperature was 85° C. Samples were centrifuged, sterile filtered (0.2μ), and then diluted 1/5 with deionized water. Seven samples were taken at various times during the 194 hour fermentations (see Data, pages 1 and 2).

Results and Discussion

The media additives; phosphatidylcholine/albumin complex or protective agent (PA), soy flour, and yeast extract /peptone (YEP) 2x, have been shown in various studies to increase ethanol tolerance or fermentative capabilities. This study has attempted to show similar improvements in fermentations containing added ethanol at 8% (w/v) and using the same nutrients at recommended concentrations.

The glucose levels in all of the flasks increased from 6 to 10 g/l (see graph 1) over the fermentation period. It was difficult to discern any significant glucose consumption rate from this data. The fact that ethanol was being produced (a high of 13 g/l for the controls grown at 30° C and a low of 1.5 g/l for the soy flour media) suggests that at least some glucose was being consumed (see graph **2).** The increase in glucose concentration along with the production of ethanol implies that the rate of glucose release was faster than the rate of glucose consumption.

Ethanol levels of the controls **(flasks** 1-6) increased only slightly and may have decreased in the remaining fermentations (flasks **7-15).** This may be **due** to low water activity, **high** osmotic pressure, or absorption of ethanol from increased surface area of media additives. Several conditions may have contributed to the lack of any significant positive effect **on** ethanol tolerance for these ethanol fermentations. **Mixing** 6% soy **flour** with the corn fiber created a media with the consistency of a thick paste. **This** created an environment of very low water activity. Possibly, a water soluble extract of soy flour added to the media would prove more beneficial. The YEP media added at twice the normal concentration combined with the corn steep liquor at twice the standard concentration could have increased the osmotic pressure to inhibitory levels. Determining **an** optimal level of YEP in conjunction with corn steep liquor would resolve this question.

The low level of emulsification of the phosphatidylcholine/albumin complex might have prevented the **PA** complex from interacting properly in the fermentation. Other researchers have dissolved **similar** types of insoluble media components in ethanol or a surfactant such as tween 80 [10,11]. The fact that the conditions were more adverse for the fermentations with added media components compared to the controls **and** the fermentation performance was not compromised to a large **degree** suggests that there may have been some improvement in fermentation capability or ethanol tolerance. One other note is that some of these media additives contain fatty acids that have been shown to be toxic for slightly aerobic fermentations at optimal levels for anaerobic fermentations[5]. These fermentations are not completely anaerobic and it is possible that this created some inhibitory conditions. Finally, the shock from starting at **8%** (w/w) ethanol might have been too high, thus diminishing the effectiveness of the supplements. Additional experiments might need to be started at **a** lower initial ethanol concentration.

Conclusions

None of the media additives showed a significant enhancement of fermentation capability in the presence of 8% (w/w) ethanol. More extreme conditions such as low water activity **and** high osmotic pressure did not seem to have a large negative impact on ethanol production. **A** number of reasons may be responsible for the **lack** of significant improvement in fermentation including variations in media preparation **and** fermentation conditions compared to that of previously described work.

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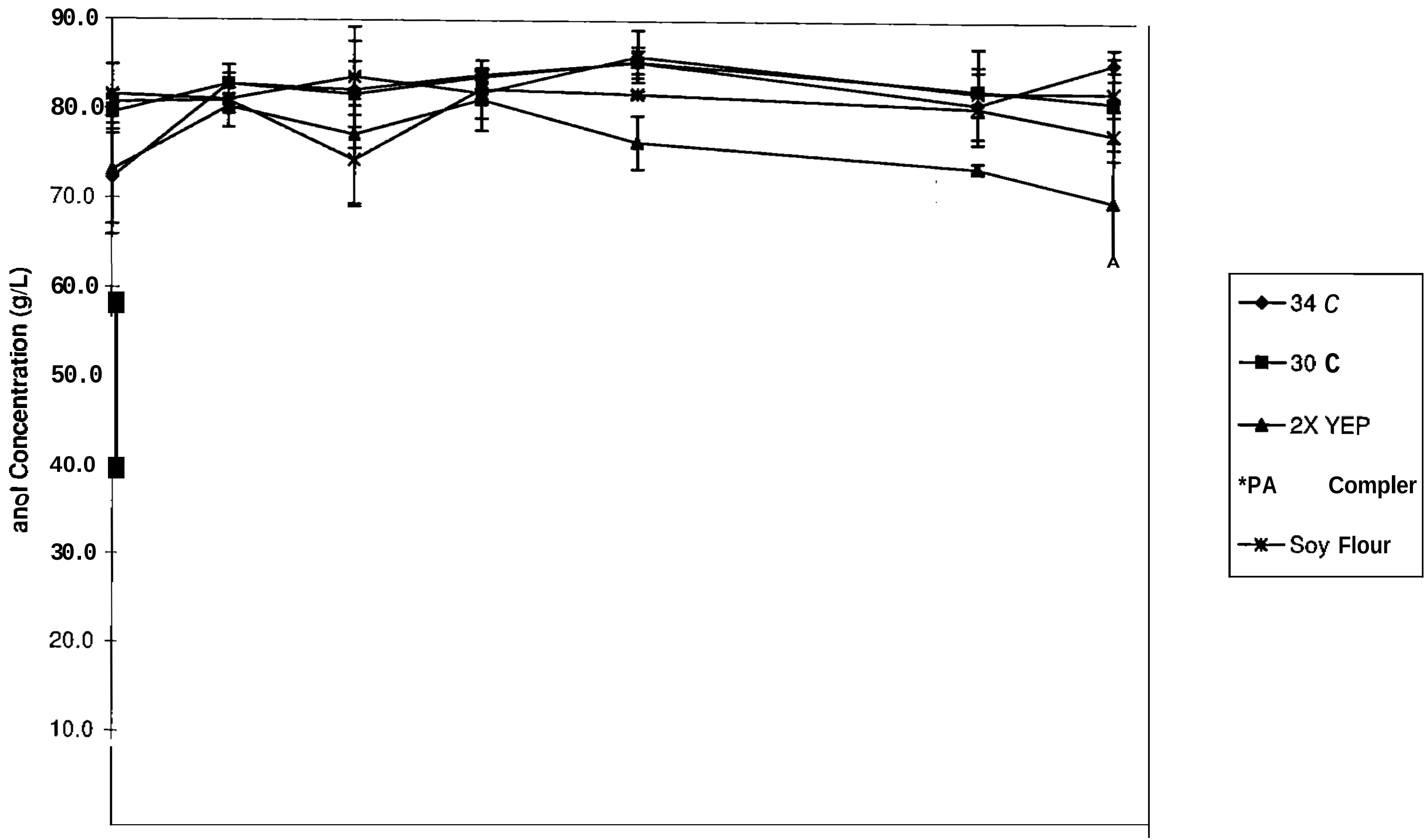
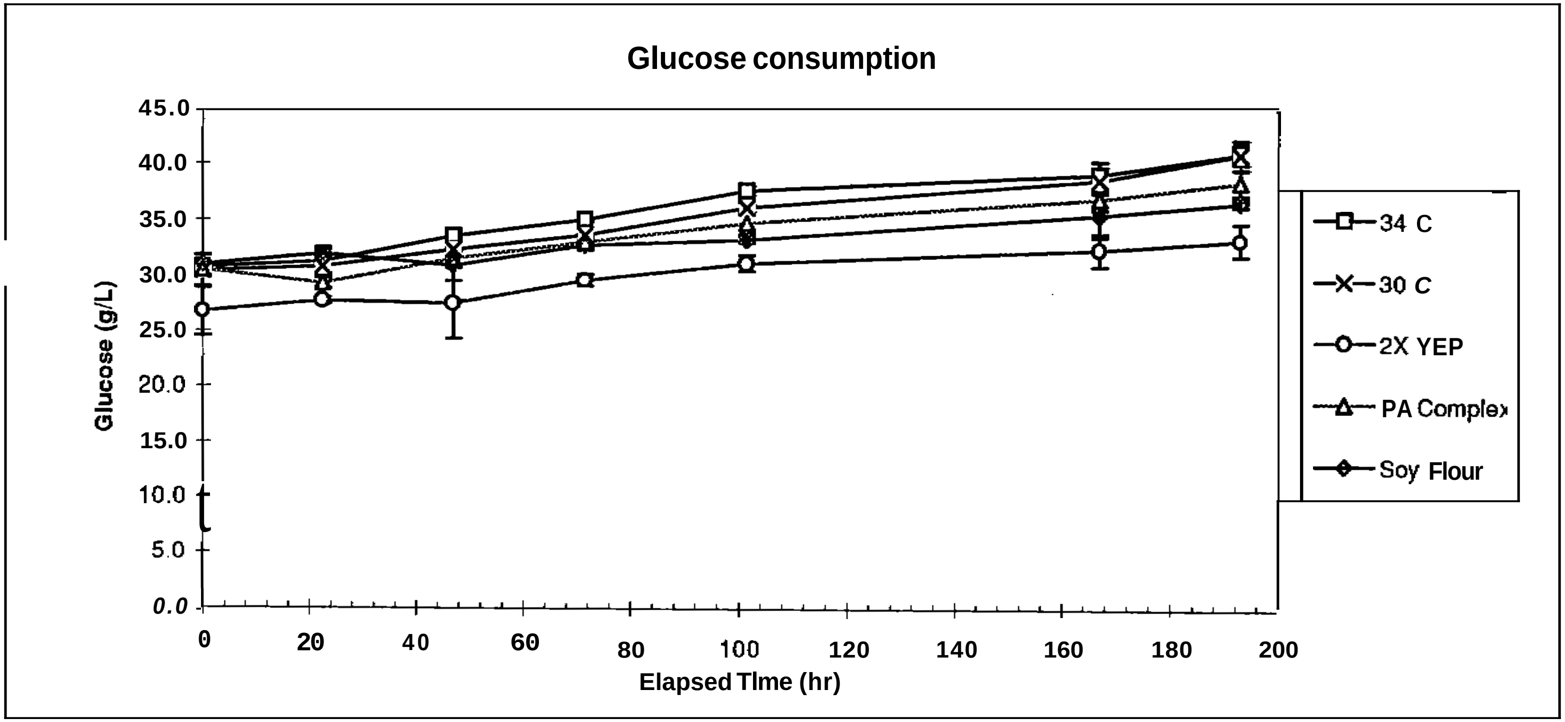
Ethanol Concentration

Ethanol Tolerance w/ L1400



Glucose Concentration



Data Sheet 2

		Ethanol Data				
Time sample	Elapsed Time	Flask 1	Flask 2	Flask 3	AVG	STDDEV
T0	0	68.1	78.2	70.6	72.3	5.3
T1	22.5	83.4	84.5	80.4	82.8	2.1
T2	47	83.1	83.2	80.1	82.1	1.8
T3	71.5	82	84.4	85.2	83.9	1.7
T4	101.5	84	84.8	87.3	85.4	1.7
T5	167	82.1	80.6	79.7	80.8	1.2
T6	193	84.1	87.3	84.6	85.3	1.7
Time sample	Elapsed Time	Flask 4	Flask 5	Flask 6	AVG	STDDEV
T0	0	81.3	80.4	76.8	79.5	2.4
T1	22.5	84	82.6	81.7	82.8	1.2
T2	47	86	84.1	74.8	81.6	6.0
T3	71.5	82.9	84.3	83.7	83.6	0.7
T4	101.5	86.7	84.1	85.4	85.4	1.3
T5	167	80.5	85.4	81.1	82.3	2.7
T6	193	83.4	75.2	84.5	81.0	5.1
Time sample	Elapsed Time	Flask 7	Flask 8	Flask 9	AVG	STDDEV
T0	0	73.2	80.4	65.8	73.1	7.3
T1	22.5	79.8	79.8	81.2	80.3	0.8
T2	47	80.8	67.9	82.9	77.2	8.1
T3	71.5	77.3	82	84	81.1	3.4
T4	101.5	73	78.1	78.2	76.4	3.0
T5	167	74.4	73.5	73.2	73.7	0.6
T6	193	74	62	73.8	69.9	6.9
Time sample	Elapsed Time	Flask 10	Flask 11	Flask 12	AVG	STDDEV
T0	0	83.8	83.2	77.7	81.6	3.4
T1	22.5	82.3	81	79.9	81.1	1.2
T2	47	82.4	89.7	78.7	83.6	5.6
T3	71.5	84.7	79.1	81.7	81.8	2.8
T4	101.5	83.3	89.1	85.7	86.0	2.9
T5	167	82.2	87	77	82.1	5.0
T6	193	84.5	73.7	88	82.1	2.5
Time sample	Elapsed Time	Flask 13	Flask 14	Flask 15	AVG	STDDEV
T0	0	81.5	80.6	79.9	80.7	0.8
T1	22.5	84	78	80.7	80.9	3.0
T2	47	61.7	84.2	77.2	74.4	4.9
T3	71.5	81.3	83.8	81.8	82.3	1.3
T4	101.5	82.2	81.5	81.6	81.8	0.4
T5	167	75.8	83.1	82.3	80.4	4.0
T6	193	83.7	79.8	69	77.5	2.8

Data Sheet 1

Glucose Data

Time sample	Elapsed Time	Flask1	Flask2	Flask3	AVG	STDDEV
T0	0	30.7	30.4	31.2	30.8	0.4
T1	22.5	31.1	32.4	30.3	31.3	1.1
T2	47	34.1	33.4	33.2	33.6	0.5
T3	71.5	35	35.2	35	35.1	0.1
T4	101.5	38	37.2	37.7	37.6	0.4
T5	167	39.7	39.9	37.8	39.1	1.2
T6	193	41.2	41.8	40	41.0	0.9

(Time sample	Elapsed Time	Flask 4	Flask 5	Flask 6	AVG	STDDEV
T0	0	28.8	31.4	31	30.4	1.4
T1	22.5	29.9	31.1	31.4	30.8	0.8
T2	47	32.5	32.4	32.1	32.3	0.2
T3	71.5	33.8	33.7	33.5	33.7	0.2
T4	101.5	36.2	35.8	36.5	36.2	0.4
T5	167	37.3	39.2	39.4	38.6	1.2
T6	193	39.8	40.6	42.4	40.9	1.3

sample	Time	Flask 7	Flask 8	Flask 9	AVG	STDDEV
T0	0	24.2	28	27.9	26.7	2.2
T1	22.5	27.4	27.8	27.9	27.7	0.3
T2	47	29.3	23.8	29.4	27.5	3.2
T3	71.5	30.1	29.1	29.6	29.6	0.5
T4	101.5	31.9	30.5	31.1	31.2	0.7
T5	167	34.1	31.6	31.5	32.4	1.5
T6	193	34.5	33.7	31.7	33.3	1.4

Time sample	Elapsed Time	Flask 10	Flask 11	Flask 12	AVG	STDDEV
T0	0	29.9	30.9	30.8	30.5	0.6
T1	22.5	28.8	29.7	29.4	29.3	0.5
T2	47	32.7	32.7	29.2	31.5	2.0
T3	71.5	32.8	33.6	32.7	33.0	0.5
T4	101.5	33.9	35.8	34.6	34.8	1.0
T5	167	36.3	38	36.5	36.9	0.9
T6	193	38	37.6	39.7	38.4	1.1

Time sample	Elapsed Time	Flask 13	Flask14	Flask 15	AVG	STDDEV
T1	22.5	32.1	31.31	32.4	31.91	0.6
T2	47	27.2	32.7	32.8	30.9	3.2
T3	71.5	32.2	33.1	33	32.8	0.5
T4	101.5	33.2	33.6	33	33.3	0.3
T5	167	33.8	37.7	35.1	35.5	2.0
T6	193	36.3	37	36.5	36.6	0.4